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# Evaluation of Prevention of DNA Damage and Induction of DNA Repair by Natural Compounds

Dissertação de Mestrado em Biotecnologia e Bioempreendorismo em Plantas Aromáticas e Medicinais
Área de Especialização em Genética Molecular

Trabalho efectuado sob a orientação de
**Professor Doutor Rui Pedro Soares Oliveira**
**Professor Doutor Bjorn Fredrik Johansson**

Outubro de 2009

**Acknowledgments/ Agradecimentos**

First, I want to express my thanks to my family and friends for all love and support given in all this years, because without them this wouldn't be possible.

I thank my supervisors, Prof. Rui Pedro Soares Oliveira and Prof. Björn Fredrik Johansson, for all aid and knowledge they shared in the development of this work.

Also, I would like to thanks all my laboratory partners, namely, Cláudia Fançony, Dário Trindade, Flávio Azevedo, Flávia Viana, Rose Marie, Rui Silva and Yolanda Delgado, since my work was easier with their help and, principally, their friendship.

Finally, I want to show my gratitude to all technical and auxiliary staff of the Department of Biology for all assistance in the realization of this work, especially Cristina Ribeiro.

**Avaliação da prevenção de danos de ADN e indução da reparação de danos de ADN por compostos naturais**


**Resumo**

As células estão permanentemente expostas a stresse ambiental (radiação e agentes químicos) e do metabolismo oxidativo nas mitocôndrias. Um dos alvos deste stresse é o ADN genómico para o qual os seres vivos desenvolveram mecanismos moleculares de reparação dos danos resultantes que envolvem enzimas como glicosilases, nucleases, polimerases e ligases. O equilíbrio entre o stresse que provoca danos de ADN e a capacidade de reparação do ADN é crucial para a sobrevivência celular. De entre estes stresses, o stresse oxidativo tem sido relacionado com muitas doenças, envelhecimento e morte celular. Uma vez detectados danos de ADN, o ciclo celular é parado o que permite a actuação destes mecanismos de reparação de danos de ADN. Esta capacidade de regular o ciclo celular em função da presença de danos de ADN é fundamental no desenvolvimento de organismos multicelulares uma vez que previne o surgimento de proliferação não controlada de qualquer célula (carcinogénese).

Neste trabalho o objectivo foi estudar o efeito protector do ADN de um extracto vegetal obtido a partir das folhas de uma árvore da espécie *Gingko biloba L.*. Os ensaios envolveram células de *Saccharomyces cerevisiae* como modelo experimental incubadas com o extracto das folhas da *Gingko biloba L.*, antes e durante o choque oxidativo provocado pelo peróxido de hidrogénio. Os danos e reparação do ADN foram determinados através do ensaio cometa, como também se calculou a sobrevivência com o ensaio de viabilidade e se mediu a oxidação intracelular pela citometria de fluxo.

Neste trabalho o ensaio cometa provou ser um método sensível e robusto na detecção dos danos no ADN nas células de levedura, tal como tem sido descrito para células animais. Os nossos resultados demonstraram a capacidade protectora do extracto da *Gingko biloba L.* contra os danos no ADN causados pelo peróxido de hidrogénio. Para além disso, o extracto protegeu as células da morte celular e reduziu a oxidação intracelular. Por fim, a actividade do extracto parece estar envolvida aos mecanismos de reparação do ADN, já que a reparação foi mais rápida quando as células foram submetidas ao tratamento com o extracto.

**Evaluation of prevention of DNA damage and induction of DNA repair by natural compounds**


## Abstract

Cells are exposed permanently to stress imposed by the environment (radiation, chemicals) and by the oxidative metabolism in mitochondria. One of the targets of this stress is genomic DNA. Cells have developed molecular repair mechanisms involving specific enzymes such as glycosylases, nucleases, polymerases and ligases, allowing efficient removal of damage. The balance between DNA damage-causing stress and DNA repair capacity is crucial for life. Oxidative stress, one of these stresses, has been related to many diseases, aging process and cell death. Once DNA damage is sensed the cell cycle is arrested so that molecular repair mechanisms can operate, or induction of programmed cell death may take place if damage cannot be repaired. These features are essential for the right development of a multicellular organism that avoids the "selfish" uncontrolled proliferation of a given cell (carcinogenesis).

In this work we aimed to study the DNA protective effect of extracts of *Ginkgo biloba L.* leaves from oxidative stress. Typical experiments involved incubation of *Saccharomyces cerevisiae* cells with *Ginkgo biloba L.* leaf extract, before and during the oxidative shock by hydrogen peroxide. Subsequent determination of DNA damage and repair by the comet assay was performed, as well as cell death estimation through the viability assay and measurement of intracellular oxidation by flow cytometry.

The comet assay proved to be a robust and sensitive technique for detection of DNA damage in yeast cells. Our results indicate that *Ginkgo biloba L.* extracts protect yeast cells against DNA damage imposed by hydrogen peroxide. In addition, *Ginkgo biloba L.* extract has protected yeast cells against cell death and has reduced intracellular oxidation. Finally, *Ginkgo biloba L.* extract possibly interferes with the DNA repair system of yeast, because DNA repair kinetics was improved when cells were exposed to this extract.

**Index**

**Abbreviations list:**

**5'-dRP**, 5' deoxyribosephosphate;

**8-oxoG**, 8-oxo-7,8-dihydroguanine;

**AP**, apurinic/apyrimidinic;

**BB**, bilobalide;

**BER**, base excision repair;

**$H_2$DCF**, dichlorofluorescein;

**DPPH,** 1,1-diphenyl-2-picrylhydrazyl

**DSBs**, double-strand breaks;

**EGb 761**, standard *Ginkgo biloba L.* leaf extract;

**G**, Ginkgolides;

**GBE**, *Ginkgo biloba L.* leaf extract;

**$H_2$DCFDA**, dichlorofluorescein diacetate;

**LMA**, low melting agarose;

***N*7-meG**, *N*7-methylguanine;

**NER**, nucleotide excision repair;

**NMA**, normal melting agarose;

**OD**, optical density;

**PBS**, Phosphate buffered saline;

**REC**, recombination;

**ROS**, reactive oxygen species;

**SSBs**, single-strand breaks;

**Tg**, 5,6-dihydroxy-5,6-dihydrothymine;

**TLS**, translesion synthesis;

**TTLs**, terpene trilactones;

**UV**, ultra-violet;

## 1. Introduction

### 1.1. DNA damage

The DNA integrity and stability of an organism is fundamental for survival, but even under the best of circumstances DNA is continuously damaged by endogenous and exogenous genotoxic agents [1]. Genotoxic agents are those substances causing damage to the genetic material. These agents are structurally diverse and range from simple compounds such as methylating and alkylating agents to bulky DNA damaging agents [2]. The outcome of DNA damage is normally adverse, resulting in several types of DNA lesions such as DNA base modifications, single- and double-strand breaks, and the formation of apurinic/apyrimidinic (AP) lesions, many of which result in mutations, premature aging or even cell death [3, 4 , 5].

Between the various types of DNA damage, AP sites are expected to be one of the most frequent [1]. A study indicates that normal human liver cells present a steady-state level of about 50,000 AP sites per genome [6]. In addition to being abundant, AP sites are a major threat to genetic information by blocking DNA replication and transcription [7]. AP sites can arise by spontaneous hydrolysis of the N-glycosydic bond or as a consequence of the removal of damaged or inappropriate bases by DNA N-glycosylases. Furthermore, cleavage of AP sites by AP endonucleases or by DNA N-glycosylases/AP lyases results in the formation of single-strand breaks (SSBs) with 3'- or 5'-blocked ends, that cannot be used as substrates by DNA polymerases or DNA ligases [8]. Moreover, 3'- or 5'-blocked SSBs can be converted into highly toxic double-strand breaks (DSBs) after DNA replication [9]. Damaged DNA bases can occur in several ways, most importantly by methylation, oxidation and deamination of normal bases yielding a variety of lesions such as *N*7-methylguanine (*N*7-meG), 5,6-dihydroxy-5,6-dihydrothymine (Tg), uracil or the major product of oxidative damage 8-oxo-7,8-dihydroguanine (8-oxoG) [1]. The occurrence and cellular level of 8-oxoG is important in terms of mutagenesis and carcinogenesis, because it can pair with adenine and cause GC to TA transversions [10]. Mutagenic 8-hydroxyguanine lesions are present in elevated levels in aged and cancer cells. Also, inappropriate bases such as uracil can be incorporated into DNA during replication and repair. Cells have evolved a number of DNA repair and damage tolerance mechanisms that prevent and counteract the different types of DNA damage caused by constant oxidative DNA damage [4].

## 1.2. DNA repair

DNA is the only biological macromolecule that is repaired by cells. All other biomolecules are replaced, instead of repaired. So, a lot of effort has been focused on understanding how cells react to DNA damage and restore the linear DNA sequence integrity and chromatin structure. An important component of the cellular response to DNA damage is an inhibition of replicative DNA synthesis. It seems this response allows optimal repair of damage before the cell reinitiates replicative DNA synthesis and/or begins mitosis. If the damage were not repaired before initiation of S-phase, the use of a damaged DNA template during replicative synthesis could "fix" and spread mutagenic lesions that might contribute to cellular transformation. The inhibition of replicative DNA synthesis after DNA damage may be a critical step in avoiding the progressive increase in genomic changes that characterizes neoplastic transformation [11]. Nevertheless, the most important components of the cellular response to DNA damage that is incurred are the DNA repair mechanisms and the damage tolerance mechanisms. In yeast, these mechanisms include the base excision repair (BER), nucleotide excision repair (NER), recombination (REC) and translesion synthesis (TLS) pathways [4].

In yeast and mammalian cells, BER is the major DNA repair pathway for the removal of endogenous DNA damage. Although similar, BER in yeast and mammalian cells has significant differences [1, 4]. In the course of the BER route in higher eukaryotes, AP sites are recognized and incised by an AP endonuclease yielding SSBs with a 5’-deoxyribosephosphate (5’-dRP) end. Afterward, the 5’-dRP is released by the 5’-dRPase activity of DNA polymerase (short-patch BER). Alternatively, the 5’-dRP can be excised by the 5 -flap endonuclease Fen1 (long-patch BER). The resulting gaps, of one or few nucleotides, are filled in by a DNA polymerase and the stability of the DNA strand is restored after action of a DNA ligase (Fig. 1) [1, 12]. In *S. cerevisiae*, cleavage of AP sites is also initiated by an AP endonuclease. Then, other enzymes take action: Rad27 has a key role in the removal of the 5’-dRP, while Pol2 (Polε) in the DNA repair synthesis and finally Cdc9 in the ligation step [1].

Nucleotide excision repair (NER) represents the most important repair system, in both prokaryotes and eukaryotes, adapted to remove a large variety of DNA lesions, particularly those that deform the DNA helix; thus, it functions in the removal of damage induced by ultra-violet light (UV), DNA intrastrand and interstrand crosslinks,

and a variety of other DNA lesions [13]. NER pathway consists in the incision of the damaged DNA strand on both sides of the lesion, resulting in the removal of damage in an oligonucleotide fragment (25-30 oligonucleotides), followed by repair synthesis and ligation steps (fig. 1).

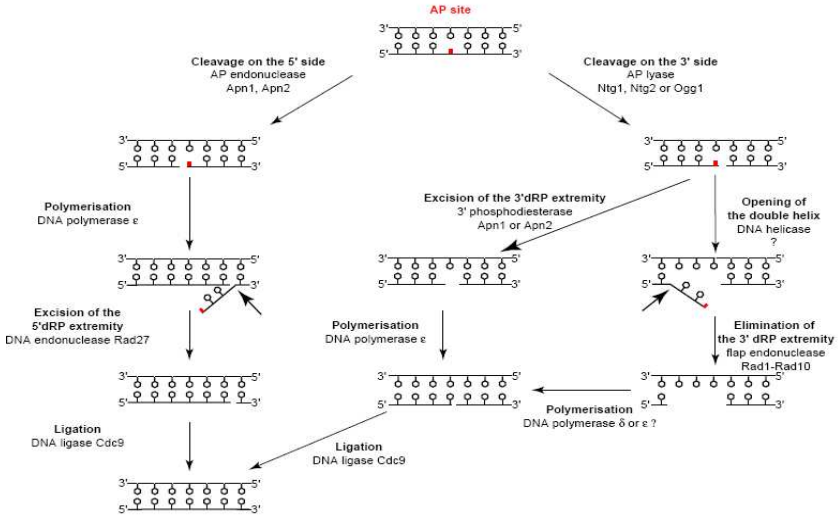


**Figure 1** - Illustrative representation of BER and NER of AP sites in S. cerevisiae [1]. *Left*: BER pathway *Right*: NER pathway

While both BER and NER are DNA repair pathways, owing to their abilities to remove and replace DNA damage, recombination and translesion synthesis are known as bypass/tolerance mechanisms since neither may actually remove DNA lesions [4].

In *S. cerevisiae*, DNA repair mechanisms cooperate to remove or tolerate AP sites and derived DNA lesions. Importantly, inactivation of the BER and NER pathways mediated by Apn1, Apn2 and Rad1–Rad10 cause early death at the stage of micro-colonies [14]. This result strongly suggests that the burden of spontaneous AP sites is incompatible with life in absence of DNA repair. This information not only reveals the importance of DNA repair pathways, but also, implies that DNA repair has emerged in the beginning of the path of evolution, allowing large genome organisms to survive. Moreover, spontaneous AP sites and derived lesion in yeast can result in genetic instability, which can mimic steps in the course of the cancer process in higher eukaryotes.

## 1.3. Oxidative stress and aging

Reactive oxygen species (ROS) are generated by normal metabolic processes in all oxygen utilizing organisms and they mediate a necessary and vital role in signal transduction [15, 16]. However, the consequences of ROS exposure are generally regarded to be undesirable [4]. ROS, such as the superoxide radical ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide ($H_2O_2$), pose a significant threat to cellular integrity. In the presence of redox-active metal ions, such as $Fe^{2+}$, $O_2^{\bullet}$ and $H_2O_2$ can undergo Fenton chemistry, generating the extremely reactive $^{\bullet}OH$ that efficiently damages DNA [17]. This free radical attacks almost all cell components: DNA, protein and membrane lipids [4, 15, 18, 19, 20]. However, downstream targets of ROS have remained largely unknown [19]. Elevated levels of ROS and the DNA damage it produces contribute to genetic instability. ROS are formed through both endogenous and exogenous routes; therefore cellular exposure to ROS is unavoidable. The majority of endogenous ROS are produced through leakage of these species from the mitochondrial electron transport chain that diffuse out freely through membranes and attack other cellular components [4]. It is estimated that about 1-2% of the total oxygen consumed by mitochondria is transformed into $O_2^{\bullet}$ [21]. The $O_2^{\bullet}$ generated is quickly converted to $H_2O_2$, which is the principal cellular mediator of oxidative stress. This conversion occurs either spontaneously, or enzymatically, catalyzed by superoxide dismutases [17]. In addition, cytosolic enzyme systems, including NADPH oxidases, and by-products of peroxisomal metabolism are also endogenous sources of ROS. Generation of ROS also occurs through exposure to numerous exogenous agents and events including ionizing radiation (IR), UV, cytokines, growth factors, chemotherapeutic drugs, environmental toxins, hyperthermia and macrophages during the inflammatory response [4]. Given the broad range of ROS sources and the highly reactive nature of many of these species, it is not surprising that ROS have been implicated in a number of disease states like cancer, neurodegenerative and cardiovascular disease as well as the aging process or cell death [4, 15, 18, 19, 21, 22, 23].

This continuous flux of oxygen radicals is normally controlled by endogenous scavenger cellular mechanisms in the cell that are highly conserved [5, 23]. These mechanisms consist of proteins involved directly in regulating redox balance or repairing effects of oxidative stress, including the well known components of the

glutathione- and thioredoxin-dependent reduction systems, superoxide dismutase, catalase, and the pentose phosphate pathway, [18, 23]. Another mechanism is a transcriptional response centred around recruitment of the “redox-specific” transcription factors to promoters of these and other antioxidant response genes [5]. However, in many conditions these protective mechanisms are overburdened (e. g. ROS accumulation exceed the cell antioxidant capability) or altered, promoting the disturbance of cell homeostasis. This is when the oxidative stress takes place and supplementary antioxidants may be required for cytoprotection.

The biological process of aging, while not fully understood, is clearly associated with an increase over time in levels of molecular damage, which contributes to increasing pathology and mortality at the organism level. The free radical theory of aging, states that ROS is the origin of oxidative damage of cell components, causing aging and consequently, cell death [17, 25]. On the other hand, the mitochondrial theory of aging, is more specific, and supports that ageing is a result of damage accumulation in mitochondrial DNA [17]. Hence, aging can be assumed as a multifactorial phenomenon characterized by a time-dependent decline in physiological function, which is connected with an accumulation of defects in metabolic pathways [17]. Accordingly, studies have demonstrated that replicative senescence of yeast cells involves a collapse of the antioxidant defence mechanisms and accumulation of oxidative damage to proteins and DNA [26] . Aging is a risk factor to cause a number of diseases like cancer. Aging and cancer share some characteristics as accumulation of genetic defects, dysfunctional redox control and impaired metabolic regulation [27].

*Saccharomyces cerevisiae* has served as a model of cellular aging more than 50 years. Two different types of aging have been studied in yeast: replicative and chronological. Yeast’s replicative age is a measure of the number of divisions a mother yeast cell can undergo, which is analogous to replicative age of various mammalian cells. On the other hand, chronological age measures the time a yeast cell can survive under nonproliferative conditions and is related to G0 mammalian cells [28].

## 1.4. Phytochemicals that prevent DNA damage and/or oxidative stress and/or aging

Nowadays, a global resurgence of interest in herbal medicine and natural compounds comes up. The importance of herbals is becoming recognised by developed countries. As a result, the use of complementary or alternative medicine has increased immensely, with more and more people believing in its benefits. Countries are now regulating the quality and benefit effects and licensing the sale of herbal products. Besides, herbs are still found in 40% of prescription drugs worldwide. In addition, this interest has increased considerably due to the need to replace synthetic antioxidants, which are being restricted by their carcinogenicity [29].

Epidemiological studies suggest that a regular consumption of fruits, vegetables and whole grains is related with reduced risks of developing chronic diseases such as cancer and cardiovascular diseases [21, 27]. This association has been partly attributed to the presence of a variety of non-nutritive phytochemicals naturally occurring in plant-based foods [27, 29]. Phytochemicals have been selected throughout the evolution and stored in plant, which confer the ability to defend themselves against pathogens and predators or act as signal compounds. Often specific to one species or a larger group of species, they contribute to give the plant its own individuality. Mammals have also evolved alongside plants, depending on some of them as staple food. Mammals have thus been exposed to a variety of phytochemicals for millions of years. Through the careful selection of plant species for food, they have avoided most phytochemicals that cause severe toxicity. However, a number of phytochemicals present in our foods still affect human health on a long-term basis positively or negatively. More than five thousand individual phytochemicals have been identified in food and beverages [30]. The challenge these days is to interpret the complex relationships between phytochemicals present in the human diet and health, taking into account both the highly diversity of their chemical structures and the complexity of their metabolic effects [30].

Chemistry of natural products is a research field with high potential. The high chemical diversity of phytochemicals suggests that they can affect a wide array of physiological functions and metabolic pathways. Most phytochemicals present in the human diet are clearly different from drugs specifically designed to interact with a specific target. Each phytochemical molecule most likely interacts with more than one

molecular target, therefore influencing different signalling pathways and the expression of a large variety of genes and modulating various metabolic pathways [16, 30]. They can be classified into several main groups: polyphenols, terpenoids, alkaloids and other nitrogen compounds, carbohydrates and lipids. Numerous reports have highlighted the free radical scavenging properties of some of those compounds, due to the fact that free radicals have been related to the development of cancer, diabetes, atherosclerosis, inflammation, and premature aging [21, 30]. The free radical scavenging property is related to antioxidant activity, as well to antigenotoxic and antiaging. So, antioxidants are compounds that can delay or prevent the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidizing chain reactions [29] and, consequently, preventing the genotoxicity and aging of cells. However, other natural compounds appear to have novel and unique actions against very specific pathways (Kapoor, Dureja et al. 2009).

Numerous natural products extracted from plants have been used as therapeutic agents, including *Ginkgo biloba* leaf extract (EGb 761), which is among the best-selling herbal medications worldwide [31]. The extract contains 22-27% flavone glycosides, 5-7% terpene trilactones (ginkgolides - G - and bilobalide - BB), less than 5 mg/g ginkgolic acids and approximately 7% proanthocyanides, and other uncharacterized compounds. Bilobalide is the major terpene and accounts for about 2.9% of the total EGb 761 preparation [23, 31, 32]. Although, all constituents contribute to overall pharmacological effect of Ginkgo extracts, flavonoids and terpene trilactones (TTLs) are believed to be responsible for most of the pharmacological properties of *G. biloba* extracts [32, 33].

The main unique compounds of the extracts are the TTLs, that is, ginkgolides and bilobalide. They are found exclusively in *G. biloba L.* tree. The ginkgolides are diterpenes with a cage skeleton consisting of six five-membered rings: a spiro (4, 4) - nonane carbocyclic ring, three lactones, and a tetrahydrofuran ring. Furthermore, they contain an unprecedented *tert*-butyl group. The ginkgolides vary only in the number and positions of their hydroxy groups (Fig. 2) [31, 32]. Bilobalide also contains three lactones and the characteristic *tert*-butyl group, as well as a secondary and a tertiary hydroxyl group, but only one carbocycle (Fig. 2) [31]. Ginkgolides are relatively stable substances, which contain a large number of functional groups and, therefore, possess high melting points (300 ºC) [32]. Also, they are stable to light, humidity and easily dissolved in dilute alkali and recovered quantitatively on subsequent acidification.

Specific solubility data on BB are not available but its polarity and solubility appears to be similar to those of the ginkgolides. An exception is its instability above pH 7.53 [32]. Flavonoids have two main chemical structures: aromatic rings with double bonds and phenolic hydroxyl groups. So, flavonoid components have high affinity to react with hydroxyl radicals to form an additional product, and thus scavenge the hydroxyl radicals directly. The phenolic hydroxyl groups possibly chelate the ferrous cation and decrease the formation of hydroxyl radicals generated from $H_2O_2$ and $FeSO_4$. The protective effect of EGb761 on apoptosis may be the effect of both scavenging of hydroxyl radicals directly and chelating iron to inhibit Fenton reaction indirectly [22].

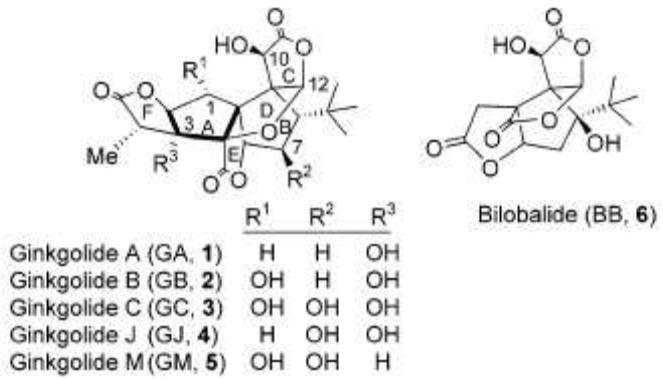


**Figure 2 -** Structure of five ginkolides and bilobalide [31].

Investigation of the effects of the individual components of *G. biloba* extract is essential for providing, through scientific documentation, the potential therapeutic effects of *G. biloba*. A major concern is the bioavailability of these components. It is assumed that the bioavailability of flavonoids is very low, whereas TTLs are nearly completely bioavailable. This further underscores the importance of TTLs when looking into effects of *G. biloba* constituents [31]. Although, both flavonoid and ginkgolides are involved in the free radical-scavenging and antioxidant effects of EGb 761 which decrease tissue levels of reactive oxygen species (ROS) and inhibit membrane lipid peroxidation [34].

Besides antioxidant activity, *Ginkgo biloba* extracts have been suggested by several studies to possess another numerous pharmacological effects, mainly, on the central nervous system including improvement of memory, antiasthmatic, decrease in cerebral insufficiency, increase in cerebral blood flow and circulation, regulation of

gene expression and to have beneficial effects to patients of Alzheimer's disease [31, 33, 34, 35]. Fundamental and clinical studies, conducted both in vitro and in vivo, support these beneficial neuroprotective effects of EGb 761 and link them to TTLs [34]. In addition, EGb 761 has been extensively used as a medicine in cardiovascular and cerebrovascular diseases [34, 36, 37]. Also, this extract is among the most prescribed medications in Germany and France for the treatment of dementia-related symptom [31].

Finally, it is very important to regard the dose-response relationships of chemical treatments. That is, beneficial effects of a treatment that at a higher concentration is damaging [16, 38]. This concept was defined Hormesis by Goldman in 1956 [39]. In one form of hormesis, sublethal exposure to stressors induces a response that results in stress resistance. The principle of stress-response hormesis is increasingly finding application in studies of aging, where hormetic increases in life span have been observed in several animal models [38].

### 1.5. Methods for DNA damage/repair assessment

DNA damage has been studied in a variety of organisms including bacteria, cyanobacteria, phytoplankton, macroalgae, plants, animals and humans. It can be spontaneous or environmental and affects all living cells in many ways [1, 40]. At present there are some methods available for detecting different kinds of DNA damage, each with its advantages and disadvantages such as comet assay, polymerase chain reaction (PCR), halo assay, TUNEL assay, flow cytometry, FISH, HPLC-electrospray tandem mass spectrometry and GC-MS.

Comparative to other methods, the advantages of the comet assay include its demonstrated sensitivity for detecting low levels of DNA damage (one break per 1010 Da of DNA) [41], requirement for small number of cells (~10,000) per sample, flexibility to use proliferating as well as nonproliferating cells, low cost, ease of application, and the short time needed to complete a study. In addition, it can be conducted on almost all type of cells. The data generated at single cell level allow for robust types of statistical analysis. On the other hand, the comet assay has its own limitations. Aneugenic effects, which may be a possible mechanism for carcinogenicity and epigenetic mechanisms of DNA damage such as effects on cell-cycle checkpoints, are not detected. Other disadvantages are single cell data (which may be rate limiting),

small cell sample (leading to sample bias), technical variability, and interpretation. Also, the variability between cells, cultures, animals as well as use of various image analysis systems or visual scoring and use of different comet parameters (e.g., Olive tail moment and tail (%) DNA), are the other factors contributing to inter-laboratory differences in the results [41]. However, its advantages far outnumber the disadvantages, and for this reason, it has gained wide acceptance as a valuable tool in fundamental DNA damage and repair studies [41, 42], genotoxicity testing, [42, 43], and human biomonitoring [41, 42, 44].

Ostling and Johanson (1984) were the first to quantify DNA damage in cells using a microgel electrophoresis technique known as “single cell gel electrophoresis or comet assay”. In this method, the cells are trapped in an inert agarose microgel on a microscope slide, deproteinized by incubation with a lysing solution, and then electrophoresed. DNA staining with a fluorochrome reveals a comet with a head and a tail of chromatin in the direction of the positive pole of the electric field. Cells with DNA breaks have a higher tail and/or greater DNA concentration in the tail. However, the neutral conditions they used, allowed the detection of only DNA doublestrand breaks. Later, the assay has been modified at various steps (lysis, electrophoresis) to make it suitable for assessing various kinds of damage in different cells [42]. So, Singh et al. (1988), adapted the assay to alkaline conditions, which led to a sensitive version of the assay that could assess both double- and single-strand DNA breaks as well as the alkali labile sites expressed as frank strand breaks in the DNA [41, 44]

The comet assay is now a well-established, simple, versatile, rapid, visual, and a sensitive, extensively used tool to assess DNA damage and repair quantitatively as well as qualitatively in individual cell populations [41, 42]. Some other lesions of DNA damage such as DNA cross-links (e.g., thymidine dimers) and oxidative DNA damage may also be assessed using lesion-specific antibodies or specific DNA repair enzymes in association with the comet assay.

### 1.6. Biological problem, strategy and methodology of this work

New chemicals are being added each year to the existing burden of toxic substances in the environment and this constitutes a serious risk for man and environment. Some of the chemicals, e.g., pesticides and heavy metals, may be genotoxic to the sentinel species and/or to non-target species, causing deleterious effects

in somatic or germinative cells. Procedures which help in hazard prediction and risk assessment are important to assess the genotoxic potential of chemicals before their release into the environment or commercial use as well as DNA damage in flora and fauna affected by contaminated/polluted habitats [41]. Sentinel species are the first to be affected by adverse changes in their environment. Determination of DNA damage using the comet assay in these indicator organisms would thus provide information about the genotoxic potential of their habitat at an early stage. This would allow for intervention strategies to be implemented for prevention or reduction of deleterious health effects in the sentinel species as well as in humans.

To the best of our knowledge, there are few reports for comet assay successful application on yeast cells. However, it would be advantageous to apply it to *Saccharomyces cerevisiae*, because this is a fast growing organism and its cultivation is cheap and easy to handle. Besides, according to the new EU regulation, REACH (Registration, Evaluation and Authorisation of Chemicals), it is recommended that the use of higher animals in chemical testing should be reduced [45]. Therefore, using *S. cerevisiae* could resolve this problem. Also, higher eukaryotes and yeast exhibit striking similarities in the molecular mechanisms of fundamental cellular processes, such as transcription, translation, replication, and DNA repair. About 30% of the human disease-associated genes significantly match yeast genes and, in contrast to human cells, yeast genes can be easily manipulated through molecular biology techniques [21]. For these reasons, the yeast has become a valuable tool for studying the eukaryotic cell and it has also been used as a test organism for estimating the mutagenic or antimutagenic potential of different chemicals and natural compounds [10, 45, 46]. Besides, mutant strains of yeast that contain specific gene deletions are available for all the known eukaryotic DNA repair pathways.

In this work we aim to study the protective effect of plant extracts obtained from leaves of a *G. biloba L.* tree. Typical experiments involved incubation of *S. cerevisiae* cells with GBE, before and during oxidative stress induced by $H_2O_2$, which produces base oxidation and single-strand breaks, mediated by the highly reactive hydroxyl radicals [46]. In addition, we adapted the comet assay to measure DNA repair in cells with DNA affected by $H_2O_2$ and pre-incubated with GBE. Finally, we estimated the GBE protection on viability of yeast cells treated by $H_2O_2$ and the intracellular oxidation by flow cytometry. With this study we intend also to evaluate the utility of the use of yeast cells as experimental model in the screening of natural compounds for their

antigenotoxic effect. Our results suggest that GBE has an activity in DNA protection against oxidative stress and in induction of DNA damage repair. The mechanism of action could involve direct scavenging of ROS and induction of oxidative stress response and DNA damage repair mechanisms.

## 2. Materials and Methods

### 2.1. Yeast strain, media, and growth conditions

In this work, the haploid *Saccharomyces cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) [47] was used in all experiments. Stock cultures of this strain were maintained on solid YPD medium (1% yeast extract, 2% peptone, 2% glucose and 2% agar) at room temperature. Cells were grown on liquid YPD medium (lacking agar), using an orbital shaker at 30ºC and 200 rpm, with a ratio flask volume/medium of 2/1.Yeast growth was monitored by optical density at 600 nm ($OD_{600}$).

### 2.2. Plant material and extract preparation

In this study, the plant material used were *Ginkgo biloba L.* leaves. The leaves were collected at autumn (October) from a specimen located in an urban area of Braga, Portugal. *G. biloba L.* extract (GBE) preparation was performed as described by Ding and co-workers [48]. *Ginkgo biloba* leaves were washed with deionized water and cut to exclude petioles and air-dried at room temperature. Subsequently, leaves were pulverized with a pestle into a fine powder and stored in closed glass bottles until use for extraction.

Five grams of powder were transferred into 200 mL polypropylene centrifuge tubes, 30 mL of boiling deionized $H_2O$ was added to the tubes, the mixture was heated in a water bath at 100ºC for 5 min, and the supernatant was obtained after centrifugation at 2000 *g* for 15 min. This process was repeated once. The supernatants were pooled, cleaned by filtration with 0.2 µm and 0,5 µm filters, pH adjusted to 6.5 with NaOH and stored at -20 ºC.

2.3. Comet assay

*Saccharomyces cerevisiae* cells from stock culture were removed with an inoculation loop, suspended in 10 mL of liquid YPD medium (pre-inoculum) and incubated overnight at 30 ºC, 200 rpm. An appropriate volume of the pre-inoculum was diluted to obtain a 25 mL culture with an $OD_{600}$ 0.1 and incubated under the same conditions until $OD_{600}$ 0.4–0.8. Cells were harvested by centrifugation at 5000 rpm, 4ºC for 2 min and washed twice with the same volume of deionized $H_2O$ at 4ºC. The pellet was suspended in the same volume of S buffer (1 M sorbitol, 25 mM $KH_2PO_4$, pH 6.5) at 4ºC. Cells, from 1 mL suspension, were obtained by centrifugation at 15300 rpm, 4ºC for 2 min, resuspended in lyticase buffer (2mg/mL lyticase, 500 µL S buffer 2x, 300 µL deionized $H_2O$ and 50 mM β-mercaptoethanol) and incubated at 200 rpm and 30ºC for 30 min in order to obtain spheroplasts. Eighty µL of the suspension were distributed by each aliquot (number of aliquots = number of slides). Spheroplasts were collected by centrifugation at 15300 rpm, 4ºC for 2 min and the pellet was resuspended in 80 µL low melting agarose (LMA) 1.5 % (w/v in S buffer) at 35 ºC. The mixture was applied onto glass slides coated with 0.5 % normal melting agarose (NMA) (w/v) and covered with cover slips.

After this point all the tasks were performed at 4 ºC. The cover slips were removed and 300 µL of the oxidant solution (10 mM $H_2O_2$) was applied on each gel. After an incubation of 20 min, the slides were washed with S buffer for 5 min and submerged in the lysing buffer (30 mM NaOH, 1 M NaCl, 0.05 % (w/v) laurylsarcosine, 50 mM EDTA, 10 mM Tris-HCl, pH 10) for 20 min. After cell lysis, the slides were washed three times with the electrophoresis buffer (30 mM NaOH, 10 mM EDTA, 10 mM Tris-HCl, pH 10) for 20 min. Electrophoresis was carried out with electrophoresis buffer for 10 min at 0.7 V/cm. Then, the gels were neutralized with 10 mM Tris-HCl buffer, pH 7.4 for 10 min and samples were fixed, first with ethanol 76 % and subsequently with 96 %, both for 10 min. Finally, the slides were dried at room temperature or in a laminar flow chamber and were visualized immediately or stored at 4 ºC until visualization. Before analysis in fluorescence microscope, the gels were stained with 10 µL of 10 µg/mL ethidium bromide.

2.3.1. Treatments with GBE

In pre-treatment experiments, spheroplasts were suspended in 80 µL of GBE diluted in S buffer, as indicated in results, so that osmotic protection by 1 M sorbitol is maintained, and were incubated at 30 ºC for 20 min. After incubation, spheroplasts were collected by centrifugation at 15300 rpm, 4 ºC for 2 min, washed with 80 µL S buffer and incorporated in LMA according to the comet assay procedure (see above). In simultaneous GBE/$H_2O_2$ treatments, spheroplasts were incubated in mixtures of GBE and $H_2O_2$ (see above) for 20 min at 30 ºC. The described procedure of the comet assay was followed afterwards.

2.3.2. DNA Repair

After an incubation of 20 min with the $H_2O_2$ solution, the slides were washed with S buffer for 5 min and 50 µL of GBE, diluted with S buffer as stated in Results, was applied on each gel and covered with cover slips. The slides were incubated at 37 ºC and washed with S buffer for 5 min at different time points. A control with S buffer replacing GBE was included in the experiment. The described procedure of comet assay was followed afterwards.

2.3.3. Images analysis

The images were obtained by fluorescence microscopy (Leica DMB 5000, black and white camera) with 400X magnification. Fifty comets per slide were analyzed with the CometScore software for analysis of comets. The parameter used to quantify DNA damage was tail length.

2.4. Viability assay

*Saccharomyces cerevisiae* cells from stock were removed with an inoculation loop and suspended in 10 mL of liquid YPD medium (pre-inoculum) and incubated overnight at 30ºC, 200rpm. An appropriate volume of the pre-inoculum was diluted to obtain a 25 mL culture with an $OD_{600}$ 0.1 and incubated under the same conditions until $OD_{600}$ 0.4. Cells were harvested by centrifugation at 5000 rpm, 4ºC for 2 min, washed

twice with the same volume of deionized $H_2O$ at 4ºC and resuspended in the same volume of S buffer. Subsequently, 100 µL of the cell suspension was harvested, serially diluted to $10^{-4}$ in deionized $H_2O$ and spread on solid YPD medium. Hydrogen peroxide was immediately added to the suspension (5 mM final concentration) and incubated at 30 ºC, 200 rpm. The same procedure for harvesting cells, serially diluting solid rich medium inoculation was followed at different time points. Plates were incubated at 30 ºC for 48 hours and the colonies counted. Survival rates were calculated as percentage of colony forming units in relation to the control situation (without $H_2O_2$).

2.4.1. Treatments with GBE

Pre-treatment with GBE was made by addition of GBE, prior to $H_2O_2$ incubation, to the cell suspension in S buffer (GBE diluted 6 fold), incubation at 30 ºC, 200 rpm for 20 min. Simultaneous GBE/$H_2O_2$ treatment was made by incubation in a mixture of 5 mM $H_2O_2$ with GBE diluted 6 fold. For both cases, cells were washed with the same volume of deionized $H_2O$ at 4 ºC and resuspended in the same volume of S buffer. After this point, the same procedure of viability assay was followed.

2.5. Flow cytometry

*Saccharomyces cerevisiae* cells from stock culture were removed with an inoculation loop, suspended in 10 mL of liquid YPD medium (pre-inoculum) and incubated overnight at 30 ºC, 2000 rpm. An appropriate volume of the pre-inoculum was diluted to obtain a 25 mL culture with an $OD_{600}$ 0.1 and incubated under the same conditions until $OD_{600}$ 0.4–0.8. Cells were harvested by centrifugation at 5000 rpm, 4ºC for 2 min and washed twice with the same volume of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4$, 1.47 mM $KH_2PO_4$, pH 7.4). The suspension was diluted to $OD_{600}$ 0.02 and 500 µL were removed for auto fluorescence measurement. Dichlorofluorescein diacetate ($H_2$DCFDA) was added (50 µM final concentration) and the cell suspension was further incubated at 30 ºC, 200 rpm for 1h in the dark. After incubation, cells were washed twice with the same volume of PBS and aliquots of 500 µL were mixed with $H_2O_2$ (10 mM final concentration) and incubated at 30ºC, 200 rpm for 20 min. Twenty thousand cells of each sample were analyzed by flow cytometry in an Epics® XLTM cytometer (Beckman Coulter) equipped with an argon-ion laser

emitting a 488 nm beam at 15 mW. Green fluorescence was collected through a 488 nm blocking filter, a 550 nm long-pass dichroic and a 225 nm band-pass filter. Data were analyzed and histograms were made with the WinMDI 2.8 software.

2.5.1. Treatment with GBE

After distribution by aliquots, cells were sedimented by centrifugation at 5000 rpm, 4ºC for 2 min and the pellet was suspended in GBE (different dilutions in PBS were used as stated in Results). Samples were incubated at 30ºC, 200 rpm for 20 min, washed twice with the same volume of PBS and treated with $H_2O_2$ as described above for flow cytometry analysis.

2.6. Statistical analysis

The experiments were done at least in triplicate and results are presented as a mean ± standard deviation (SD). One-way analysis of variance (ANOVA) was used for comparison of more than two means and Tukey´s test to multiple comparisons. All asterisks indicate differences considered statistically significant: * means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$, when compared to the respective control.

## 3. Results

3.1. GBE protects yeast cells against DNA damage by $H_2O_2$

Among many natural products with antioxidant activity is a standard *Ginkgo biloba L.* leaf extract (EGb 761), which is one of the best-selling medicinal plants worldwide. The Asian tree *Ginkgo biloba L.* was mentioned in traditional Chinese medicine and has generated scientific interest for its reputed value in treatment of memory related afflictions [48, 49]. Recent research has focused on the medicinal value of its leaves. Extracts of *G. biloba L.* were reported to be antiasthmatic, reduce cerebral insufficiency, scavenge radicals, increase cerebral blood flow and circulation, act as gene regulator, improve memory and have beneficial effects to patients of Alzheimer's disease [31, 33, 34, 35]. Antioxidant activity elicited by EGb 761 has been confirmed by several studies [19, 21, 22, 23, 33]. However, none of them refers to DNA protection

(antigenotoxic activity), prevention of DNA damage or induction of DNA repair. To our knowledge only one investigation [21] used *S. cerevisiae* cells as experimental model.

In the beginning, we started to evaluate if antioxidant activity of GBE may possibly prevent DNA damage induced by $H_2O_2$. For this, we used the comet assay applied to yeast cells as experimental model. In these experiments, yeast spheroplasts were pre-treated with various GBE dilutions in S buffer in order to maintain osmotic protection of spheroplasts, and subsequently, exposed to 10 mM $H_2O_2$ and analyzed for DNA damage. Additionally, several controls were included in this experiment: incubation with deionized $H_2O$ as negative control of the oxidative agent $H_2O_2$; incubation with S buffer as negative control of GBE before treatment with $H_2O_2$; incubation with $H_2O_2$ alone as control of this reagent; incubation with S buffer as control of the solution used in GBE dilutions; and incubation with GBE diluted 2 fold without subsequent exposure to $H_2O_2$. Control experiments indicated that S buffer and GBE do not cause damage to DNA (fig. 3). In addition, $H_2O_2$ dramatically increases comet tail length, which is not alleviated by S buffer. When yeast spheroplasts were pre-treated with GBE dilutions before exposure to $H_2O_2$, a statistically significant decrease in comet tail length was observed, when compared to the control situation (S buffer + $H_2O_2$) (figs. 3 and 4). As depicted in fig.3, a good correlation between dose of GBE and response was established.

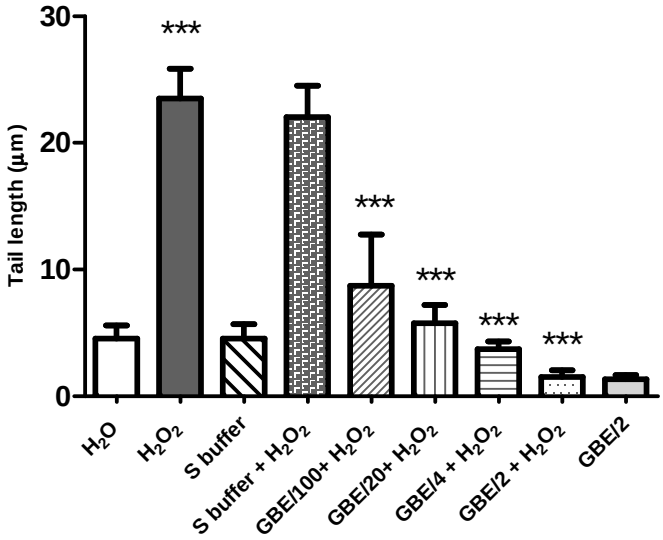


**Figure 3** – Pre-treatment of *S. cerevisiae* cells with GBE protects DNA against oxidative damage by $H_2O_2$. Yeast spheroplasts were incubated with GBE (diluted 2, 4, 20 and 100 fold) for 20 min, washed, and subsequently, incubated with 10 mM $H_2O_2$ for 20 min. DNA damage was analyzed by the

comet assay method (see Materials and Methods). Mean ± SD values are from five independent experiments (*** represents $p < 0.001$).

These results are in accordance with a previous report [22], describing that the standardized *G. biloba* leaf extract, EGb 761, attenuates oxidative damage induced by $H_2O_2$ in rat cereberal granular cells. Furthermore, GBE diluted 2 fold, alone, decreased comet tail length when compared with incubation with S buffer alone. This suggests that the *G. biloba* leaf extract may decrease background DNA damage in a given yeast population. Finally, other experiments (not shown) with higher GBE dilutions (500, 1000 and 10000 fold) suggested that only at 1000 fold dilution, GBE reached the same values of control tail length (S buffer + $H_2O_2$). In an attempt to discriminate induction of cellular DNA repair or antioxidant response mechanisms from a ROS scavenging activity, we decided to try an incubation of GBE and $H_2O_2$ at the same time.

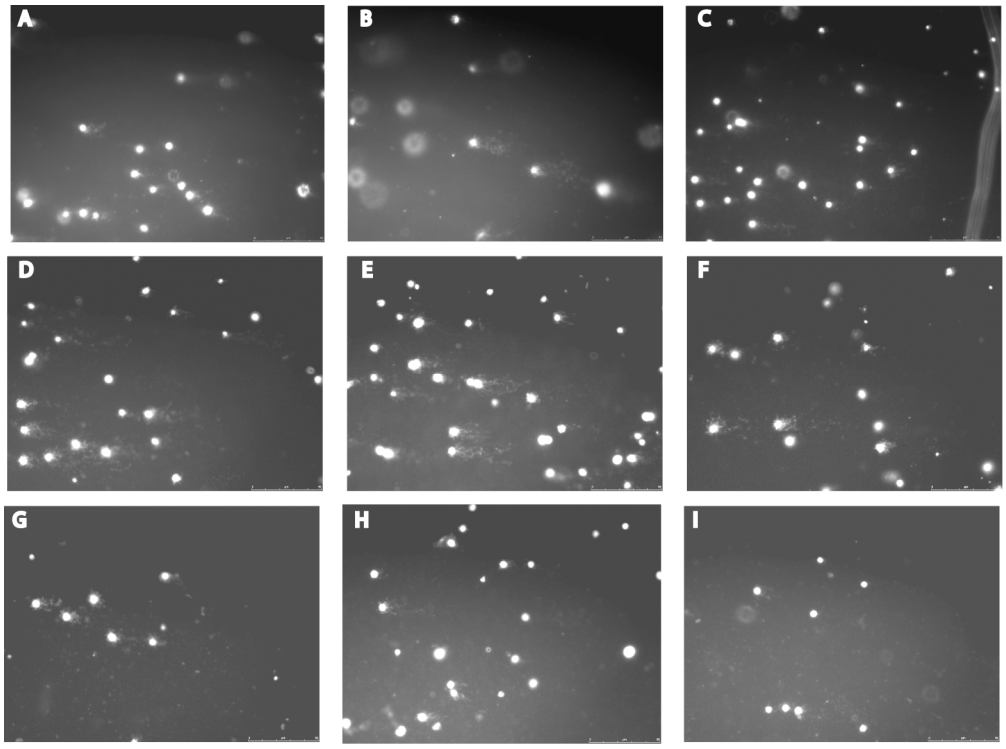


**Figure 4** – Images of the yeast comet assay after DNA labelling with ethidium bromide. A: control experiment with deionized water for cell treatment; B: cells treated with 10 mM $H_2O_2$; C: control experiment with S buffer for cell treatment; D: control experiment with incubation with S buffer before incubation with 10mM $H_2O_2$; E: incubation with GBE diluted 100 fold before incubation with 10mM $H_2O_2$; F: incubation with GBE diluted 20 fold before incubation with 10mM $H_2O_2$; G: incubation with GBE diluted 4 fold before incubation with 10mM $H_2O_2$; H: incubation with GBE diluted 2 fold before

incubation with 10mM $H_2O_2$; I: control experiment with incubation with GBE diluted 2. All images were obtained at 400X magnification.

Also, in simultaneous treatment experiments, several dilutions of GBE were made in S buffer, but this time $H_2O_2$ was added right before the incubation step, so that cells were incubated with a mixture of GBE and $H_2O_2$. Simultaneous treatment controls were included to test background DNA damage (deionized water); DNA damage induced by $H_2O_2$ alone as control of this reagent; DNA damaging activity of the solvent used for GBE dilutions (S buffer alone); DNA protective activity of the solvent used for GBE dilutions (S buffer + $H_2O_2$); and GBE damaging effect on DNA (GBE/4). Results of control experiments and of GBE activity assays were comparable, although slightly lower in absolute values, with those obtained in pre-treatment experiments (figs. 3 and 5). Data from the simultaneous incubation experiment (fig.3) suggest that GBE has a direct inactivating effect on $H_2O_2$. However, an effect on cellular adaptation mechanisms like oxidative stress reponsse and DNA repair cannot be excluded as well.

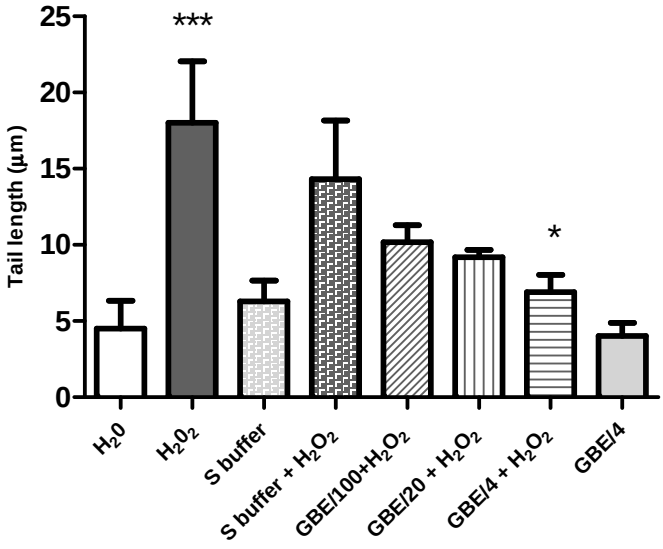


**Figure 5** – GBE can attenuate DNA damage of *S. cerevisiae* cells when incubated simultaneously with $H_2O_2$. Yeast spheroplasts were incubated with GBE (diluted 4, 20 and 100 fold) simultaneously with $H_2O_2$ (final concentration 10 mM) for 20 min. DNA damage was analyzed by the comet assay method (see Materials and Methods). Mean ± SD values are from three independent experiments (*** represents $p < 0.001$ and * represents $p < 0.05$).

We also tested the activity of the standardized EGb 761 extract by comet assay, which is made from *G. biloba* leaves and has known relative composition on flavone glycosides, terpene trilactones and ginkgolic acids, the main unique compounds of this tree. This extract is also frequently used in experiments related with *Gingko biloba L* effects. The assays were performed under the same conditions as mentioned for the pre-treatment assays of our extract. Results obtained were similar to our extract (fig. 6), which suggests that the active compounds with antigenotoxic activity are present in both preparations.

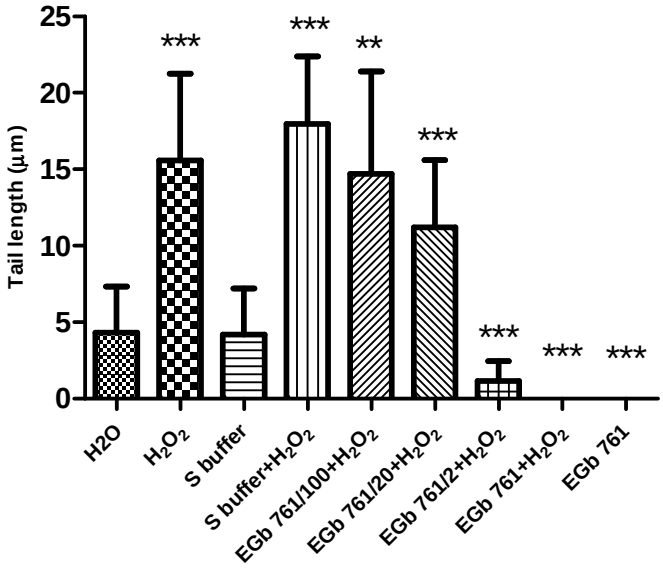


**Figure 6** – Pre-treatment of *S. cerevisiae* cells with EGb 761 protects DNA against oxidative damage by $H_2O_2$. Yeast spheroplasts were incubated with EGb 761 (diluted 2, 20 and 100 fold) for 20 min, washed, and subsequently, incubated with 10 mM $H_2O_2$ for 20 min. DNA damage was analyzed by the comet assay method (see Materials and Methods). Mean ± SD values are from five independent experiments (*** represents $p < 0.001$).

### 3.2 GBE improves the DNA repair ability in yeast cells

To investigate the possibility of an activating effect on DNA repair mechanisms by GBE, we decided to assess DNA repair kinetics using a modification of the comet assay optimized for DNA damage repair in yeast cells (Azevedo, unpublished results). DNA repair experiments consisted in provoking DNA damage in yeast cells with 10 mM $H_2O_2$ for 20 min to induce damage in DNA, and then by incubation with GBE diluted two fold in S buffer at 37 ºC up to 20 min. The control experiment consisted in

incubation with S buffer, which is the solvent for GBE dilution. As expected, comet tails length decreased during the 20 min incubation in both situations (fig. 7). However, in GBE-treated cells, comet's tails decreased more rapidly and were not detectable after 10 min incubation. These results strengthen the involvement of GBE not only in DNA damage prevention, but also in DNA repair mechanisms.

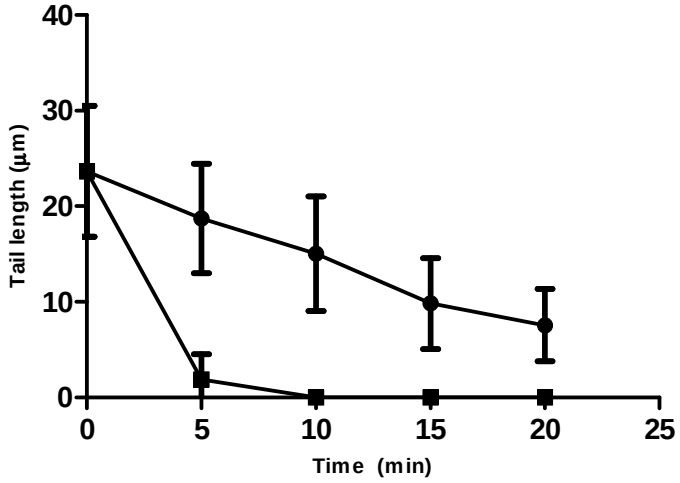


**Figure 7** – GBE increases DNA repair ability in *S. cerevisiae* cells upon damage by $H_2O_2$. Yeast spheroplasts were incubated with 10 mM $H_2O_2$ for 20 min, washed, and subsequently, incubated with GBE (diluted 2 fold in S buffer; ■) or S buffer (control; ●) for different incubation times (0, 5, 10, 15 and 20 min). Spheroplasts were washed and DNA damage was analyzed by the comet assay method. Results are the mean of three independent experiments.

### 3.3. GBE increases viability of *S. cerevisiae* cells under oxidative stress

Among published articles that report the antioxidant activity of GBE [19, 21, 22, 23, 33], only one [21] used *S. cerevisiae* cells as experimental model, but results revealed an ineffective protection by EGb 761 against *S. cerevisiae* cells death caused by *tert*-butylhydroperoxide. Those were unexpected results according the other studies and the results we obtained from the comet assay. Therefore, we decided to investigate the effect of GBE on *S. cerevisiae* cells viability under conditions of oxidative stress. This attempt aimed to complement the results obtained from comet assay in order to understand better the mechanism of action of GBE. Perhaps, the DNA damage protective effect and enhanced DNA repair capacity observed previously are correlated with a global protective effect against cell death mediated by $H_2O_2$.

In viability assays, *S. cerevisiae* cells were incubated for 20 min with GBE diluted 6 fold in S buffer, subsequently $H_2O_2$ was added to a final concentration of 5 mM and incubated at 30ºC. Aliquots of the culture were harvested at different time points, diluted and plated on rich medium in order to count colonies after 48h incubation at 30ºC. Cell death was considered as loss of viability expressed as percentage of colonies of test plates when compared to the reference plate (without toxic treatment). A control experiment without GBE and $H_2O_2$ was included so that viability of untreated and non-stressed cells could be determined. Results obtained (fig. 8) show that yeast cells when exposed to 5 mM $H_2O_2$ had a significant decrease in survival rate and after 60 min incubation no survivors were found. On the other hand, when cells were pre-treated with GBE diluted six fold, the survival rate was greater during the 200 minutes of exposure to 5 mM $H_2O_2$. As expected, cells without any treatment showed a nearly constant survival rate for 200 min incubation. These results strongly suggest that the protective activity on DNA against oxidative damage and improved DNA repair capacity are correlated with an increase in viability under oxidative stress conditions.

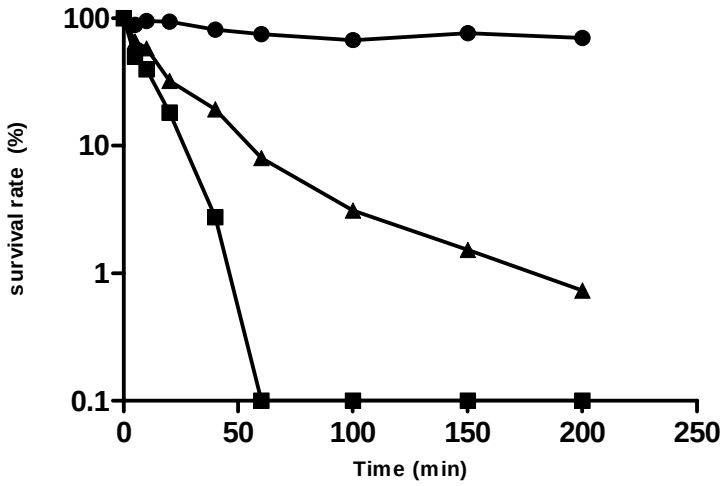


**Figure 8** – GBE increases viability of *S. cerevisiae* cells under oxidative stress. Yeast cells were incubated with GBE (diluted 6 fold in S buffer; ▲) for 20 min, washed and suspended in S buffer, and subsequently, incubated with 5 mM $H_2O_2$ for different periods (0, 5, 10, 20, 40, 60, 100, 150 and 200 min). At different time points, an aliquot was collected and, after proper dilution, cells were plated on solid YPD medium. Colonies were counted after 48h incubation at 30ºC. The same procedure was applied to cultures without GBE pre-treatment (■) and without GBE and $H_2O_2$ treatments (●). A representative experiment is presented from three independent experiments.

### 3.4. GBE decreases intracellular oxidation

Results depicted in fig. 8 strongly suggest that GBE protect cells from oxidative shock. One explanation for this is that GBE can efficiently decrease the oxidation state of cells, which would result in decreased DNA damage and increased viability upon a $H_2O_2$ insult. To test this hypothesis we investigated the intracellular oxidation of cells treated and non-treated with GBE using the redox sensitive probe dichlorofluorescein diacetate ($H_2$DCFDA). The diacetate form of dichlorofluorescein is able to diffuse freely through the plasma membrane to cells where intracellular esterases deacetylate the $H_2$DCFDA with production of dichlorofluorescein ($H_2$DCF), which does not permeate membranes. Therefore, upon incubation with $H_2$DCFDA, cells become loaded with $H_2$DCF, which is susceptible to oxidation depending on the intracellular redox environment. Oxidation turns $H_2$DCF fluorescent and able for cytometry detection. Oxidative shock by $H_2O_2$ induces an increase of intracellular oxidation and, hence, higher fluorescence in $H_2$DCFDA-loaded cells as compared to non-treated cells (fig. 9A). When cells were treated with GBE before the oxidative shock, fluorescence decreased significantly, suggesting that GBE caused an adaptation in yeast cells against oxidative shock (fig. 9A). To investigate if this adaptation protects cells exclusively from oxidative shock, we have incubated mid-log growth phase cells with GBE for 20 min and then measured fluorescence in the flow cytometer. As can be seen in fig. 9B, cells display less fluorescence after incubation with GBE, indicating that the effect of the extract includes a general decrease of the oxidation state of the cells.

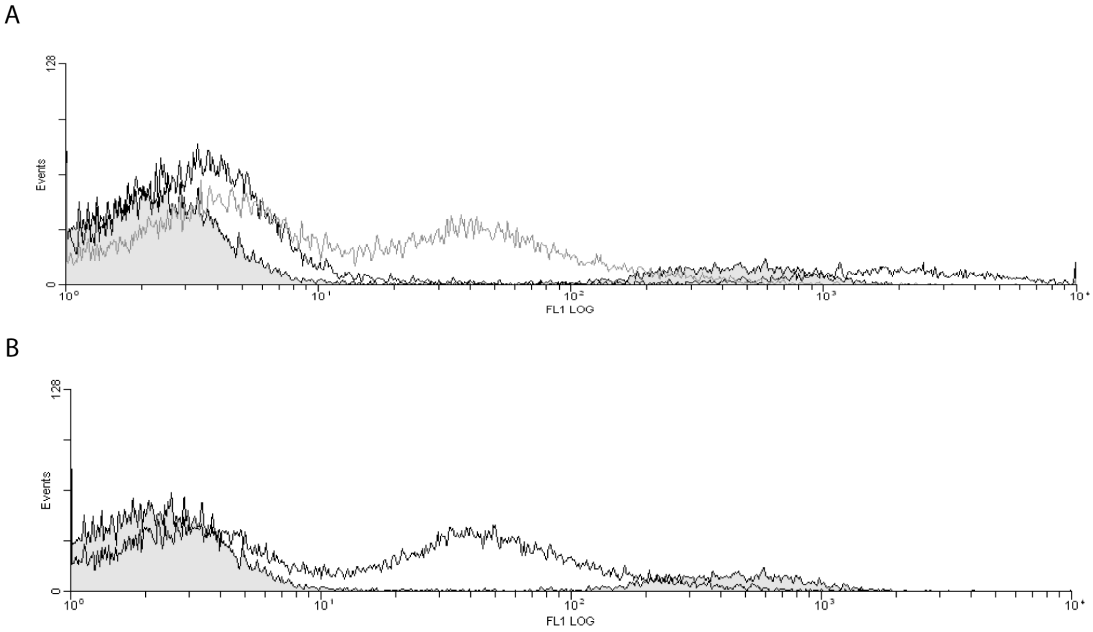


**Figure 9** – GBE decreases intracellular oxidation of *S. cerevisiae* cells. A: influence of GBE on intracellular oxidation was assessed by flow cytometry after $H_2O_2$ oxidative shock in cells loaded with $H_2$DCFDA. Solid black line: yeast cells loaded with $H_2$DCFDA after incubation for 60min in the dark; black line: cells loaded with $H_2$DCFDA and incubated with 10mM $H_2O_2$ for 20min; grey line: cells loaded with $H_2$DCFDA, incubated with GBE diluted 2 fold in S buffer for 20min and subsequently incubated with 10mM $H_2O_2$ for 20min. B: influence of GBE on intracellular oxidation was assessed by flow cytometry in cells loaded with $H_2$DCFDA. Solid black line: yeast cells loaded with $H_2$DCFDA after incubation for 60min in the dark; black line: cells loaded with $H_2$DCFDA and incubated with GBE diluted 2 fold in S buffer for 20min. Data are from a representative experiment from at least three independent experiments.

## 4. Discussion

After exploring the literature for natural compounds with antioxidant, antiaging or antigenotoxic activities we obtained a list of plants and compounds with potential interest in medicine. From these, we have looked for plants and compounds which antigenotoxic activity has not been assessed extensively. Despite the fact that there is a considerable amount of scientific papers reporting studies with *G. biloba* extracts, only a very small fraction of them concern antigenotoxic activity (**2**). So, to assess its activity we applied the yeast comet with an extract from the leaves of this tree species. Preliminary results clearly suggested a strong DNA protection activity against damage caused by $H_2O_2$. Therefore, we have decided to explore this extract to obtain important

insights on its activity mechanism. Another objective of our work was to assess *S. cerevisiae* as experimental model for antigenotoxicity tests. Our results clearly suggest that reproducible DNA damage and DNA damage repair assessment can be obtained with the comet assay in these cells. Therefore, the use of yeast as experimental model in these experiments can be a valuable option to avoid the use of higher animals in chemical testing.

The conditions used in the comet assay method were already tested and established in our laboratory in a study concerning DNA damage provoked by oxidative shock (Azevedo et al., unpublished results). We have observed a statistically significant correlation between GBE dilutions and comets tails length in pre-treatment and simultaneous treatment experiments. Hence, this dose response effect can be attributed to chemical components of the extract, which would have a scavenging effect on $H_2O_2$ and an induction of oxidative stress response and/or induction of DNA damage repair. Interestingly, pre-treatment with GBE decreased tails length in cells without $H_2O_2$ treatment (fig. 3), suggesting that GBE can protect DNA from endogenous damaging agents. In addition, to our knowledge this is the first time GBE has been involved in DNA damage repair induction and this is one of the few reports on its antigenotoxic activity.

The antioxidant effect of GBE we report in this study (fig. 9) is in accordance to what has been published [19, 21, 22, 23, 33] and with our results of increased viability on cells pre-treated with GBE and of DNA damage protection. The mechanism of action of GBE can be a direct inactivating effect on $H_2O_2$ by scavenging hydroxyl radicals and in cellular oxidative stress response, which would allow increased viability and decreased DNA damage under oxidative stress. As we show in fig. 9B, the antioxidant activity of GBE is present even without oxidative shock. This suggests that there are compounds in the GBE that can scavenge directly endogenous ROS and/or efficiently recycle endogenous scavenger cellular proteins like glutathione, thioredoxin, superoxide dismutase, catalase, and the pentose phosphate pathway. Previous reports on the biological effect of *G. biloba L.* extracts, describe diverse activities that can be explained by an antioxidant activity of GBE, These include improvement of memory, antiasthma activity, decrease of cerebral insufficiency, increase of cerebral blood flow and circulation, changed gene expression and beneficial effects in patients of Alzheimer's disease [19, 22, 31, 33, 34, 35].

In our experiments of antigenotoxic activity of GBE, we have included replicas with a standardized *G. biloba* leaf extract named EGb 761 (Schwabe Pharmaceuticals). This extract is commercially available in several countries, as Tebonin, and, according to the manufacturer, with official German authorities' approval to commercialize with indications for the treatment of progressive impairment and loss of mental capacities. Results obtained with this extract are similar with our extract, which suggests that the active compounds with antigenotoxic activity are present in both extracts. Therefore, our extract can be used for further studies aiming at the identification of the antigenotoxic compounds of GBE.

Terpene trilactones are alleged to contain the pharmacological properties of *G. biloba* extracts [32, 33]. So, separation of GBE compounds by chromatography and evaluation of their activities as isolated compounds can contribute to the identification of the pharmacologically active compounds. In this work we have successfully used the comet assay with yeast cells to assess antigenotoxic activity. Therefore, the same approach applied to the isolated compounds could help to identify the ones with this activity. The determination of the mechanism of action of the active compounds can also be studied by exploring the amenability of the genetic system of *S. cerevisiae*. By the use of available mutant strains affected in genes involved in DNA repair pathways, like *APN1*, *APN2*, *RAD1* and *RAD10* it will be possible to discriminate the DNA repair mechanism(s) activated by GBE. Preliminary results obtained in our laboratory (Azevedo, personal communication) indicate that these mutants have unaltered DNA damage repair kinetics. This can be explained by functional redundancy of these proteins, which can be overcome by the use of double mutants with various combinations of mutations. However, some of the genes involved in DNA repair are essential, or the combination of mutations are synthetically lethal, so, the alternative is the use of conditional mutants and test them under the restrictive conditions. Induction of DNA repair can be explained with an increase in enzyme activity and/or expression of the gene(s) involved in this process. Approaches involving cloning, heterologous expression and purification of the enzymes and subsequent *in vitro* testing with damaged DNA as substrate in the presence and absence of the active compound(s), can elucidate the target of these compounds. On the other hand, if increased gene expression is the mechanism, Northern analysis of the genes involved in DNA repair will help to identify gene targets. Compounds identified in GBE have potential medical use in cancer prevention due to their effect on DNA integrity. In addition, by exploring their

mechanism of activity, for instance selectively inducing enzymes involved in DNA repair, new insights could arise to shed light on the DNA repair mechanisms.

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